SPECIFIC FATS AS FACTORS IN IMMUNE PROCESSES

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The work presented in this paper is a continuation of what appeared in a previous article,[1] in which it was shown that the value of antigens in serum tests for the presence of antibody depends on fatty complexes of definite or specific configuration which represent the fatty content of the micro-organisms giving rise to the antibody. The antigens dealt with were the specific fat antigens of the red blood cells of the sheep, the gonococcus, the treponema pallida, the typhoid bacillus, and the cholera vibrio. The methods outlined in the article referred to apply to the present work and need not be repeated, but any radical departure from them will be noted and new methods given.

THE ANTIGEN OF RED BLOOD CELLS OF THE SHEEP

This antigen, consisting of the fats peculiar to these cells in the form of sodium salts, was shown to have the specific power to fix complement in the presence of its appropriate antibody. Granted that the specificity of such a reaction lies in the immune body and the antigen it should be possible by some means to produce the antibody in experimental animals by inoculating them with the antigen. Accordingly this was tried, with success.

The method of preparing the material was first given careful consideration, and then the state or condition of the material and the manner of introducing it. The idea was to imitate as closely as possible the processes of an infection and to bring the antigen in contact with the body fluids of the animal in a state at least bordering on that in which it probably exists in the red blood cells. The first effect desired was that of surface action of the antigen on the plasma of the animal comparable to the effect of the surfaces of the corpuscles themselves, the assumption being that in all cells those substances which are most active in regulating surface tension and surface permeability are found at the surface and consist largely of fat and lipoidal material. The second effect to attain was the education,

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[1] Warden, C. C.: Jour. Infect. Dis., 1918, 22, p. 133.

if one may use the word, of the plasma to those surface substances, that is, the antigen, by frequent inoculation in small quantities. The third end was to preserve proper proportions between the ingredients of the antigen and between the antigen and the plasma of the animal.

We have observed that in the fixation of complement by the fat antigens at room temperature, 37 C. and in the refrigerator, cholesterol is probably essential to accurate and clear-cut reactions, and that the best results are obtained when the relative weights of cholesterol and the fat antigen complex approximate 4:1. This ratio applies equally to the syphilitic antigen. In addition, cholesterol and its esters are constituents of nearly all cells. Accordingly, the materials first selected for antigenic purposes were:

1. An aqueous solution of the protein free salts of the specific fats of red blood cells.

2. An aqueous colloidal suspension of protein free cholesterol.

3. An aqueous suspension of typhoid bacilli which had been killed, fixed by 1% formol, washed, and kept in distilled water for a year. This suspension gave no protein reaction.

The freedom from protein of the materials employed has been determined by biuret and ninhydrin tests, by Kjeldahl determinations, and by removal of nitrogen by dialysis.

The colloidal cholesterol solution was prepared by dissolving the cholesterol in hot acetone and adding the solution drop by drop to a definite quantity of distilled water in constant motion. The strength of the solution used was such that 1 c c contained 0.001 gm. of cholesterol. This colloid is opaque, milk white and practically permanent. It can be filtered through paper and boiled without damage. It is electronegative and easily flocculated by bivalent and trivalent kations in proper concentrations. The particles are visible microscopically under high power and low illumination, have lively brownian movement and vary somewhat in form, but not greatly in size. The effect of adding the fat antigen, either in powder or solution, to this colloid is one of dispersion. In the proportion by weight of four parts of cholesterol to one of antigen there is little or no change save that the particles appear to become more rounded in contour. It may be said that this proportion of antigen is just sufficient for distribution at the surfaces of the phase cholesterol-water without producing alteration. Greater concentration, however, causes dispersion of the cholesterol particles accompanied by clearing and transparency of the mixture.

The first stages of this change may be seen under ordinary high power. It is not solution or saponification as the salts are at first neutral in reaction, and the change occurs almost instantly.

The typhoid suspension was used for its surface action, being a suspension of particles similar in toxifying power to agar.[2] The aqueous solution of the fatty salts, or antigen, is likewise electronegative. Interesting in this connection are the experiments of Gengou[3] on the agglutination and hemolysis of red blood cells by barium sulphate. We have found that this salt flocculates the corresponding fat antigen equally well, as it does also the fat antigens of the gonococcus-meningococcus group. Previous observations had shown that this salt and calcium chlorid are powerful agglutinants of both members of this group. With the materials enumerated it was believed that the desired effects mentioned earlier might be obtained.

Exper. 1.—Preliminary tests on rabbits weighing about 2,000 gm. were made to determine the toxicity of the 3 solutions on intravenous injection. Neither the aqueous solution of the fat antigen nor the typhoid suspension in doses suitable for the work produced harmful symptoms. The cholesterol colloid, however, proved fatal within 3 minutes following injections of 2 c c, with typical signs of anaphylaxis. Doses of 1 c c produced severe shock, but the animals recovered. The working dose adopted was 1 c c diluted with an equal volume of water. Guinea-pigs were found to be less sensitive.

Twelve rabbits averaging 1,900 gm. received the following intravenous inoculations daily for 3 days:

Rabbit 1: 5 mg. red blood cell antigen in 2 c c distilled water.

Rabbit 2: 3 c c typhoid suspension; then, after 30 minutes, 5 mg. blood cell antigen in 2 c c distilled water.

Rabbit 3: Duplicate of No. 2, interval of 15 minutes.

Rabbit 4: Cholesterol colloid, 1 mg. in 2 c c water.

Rabbit 5: Cholesterol colloid, 1 mg. in 2 c c water + 2.5 mg. blood cell antigen.

Rabbit 6: Typhoid suspension; 3 c c containing 2.5 mg. blood cell antigen.

Rabbit 7: Duplicate of No. 5.

Rabbit 8: Cholesterol colloid, 1 mg. in 2 c c water; then, after 20 minutes, blood cell antigen 5 mg. in 3 c c water.

Rabbit 9: Typhoid suspension, 3 c c.

Rabbit 10: Normal rabbit received no injections.

Rabbit 10A: Distilled water, 3 c c.

Rabbit 10B: Distilled water, 3 c c.

The animals were bled from the heart on the 13th day after the last injection, the serums separated and inactivated. In doses of 0.04 c c, in the presence of 0.02 c c of complement (60%) and 0.5 c c of 4% red blood cell suspension

[2] Novy and DeKruif: Jour. Infect. Dis., 1917, 20, p. 629.

[3] Ann. de l'Inst. Pasteur, 1904, 18, p. 678. Bordet: Studies in Immunity, collected and translated by Gay, 1909, p. 312.

in salt solution in a total volume of 1 c c, the serums produced hemolysis as follows:

Rabbits 2, 3 and 7, 100% hemolysis.

Rabbit 1, 90% hemolysis.

Rabbits 5 and 6, 50% hemolysis.

Rabbit 4, 25% hemolysis.

Rabbits 8, 9, 10, 10A, 10B, no hemolysis.

The experiment was then repeated by incubating together the rabbits' serums, the fat antigen, complement and salt solution for ½ hour at 37 C., followed by a second incubation after addition of red blood cell suspension. The hemolysis in this instance was the reverse of the preceding.

Rabbits 8, 9, 10, 10A, 10B, complete hemolysis, or no complement fixation.

Rabbit 4, partial hemolysis, or 75% complement fixation.

Rabbits 5 and 6, partial hemolysis, or 50% complement fixation.

Rabbit 1, slight hemolysis, or 90% complement fixation.

Rabbits 2, 3 and 7, no hemolysis, or complete complement fixation.

The serums were then tested for cross fixations with syphilitic, gonococcus, pneumococcus, and B. welchii antigens with negative results. With the typhoid antigen, Serum 2 gave 25% fixation, Serum 5, 10%. The pneumococcus and typhoid antigens will be referred to later. This experiment shows the presence of antibody in the serums of all but one of the animals that received the antigen.

Exper. 2.—Rabbits 1, 2, 4, 5, 7 and 8 received a second series of the same injections, commencing on the 17th day following the last of the first series and continued for 5 days.

Rabbit 3 received no injections, being reserved for control.

Rabbit 6 died in anaphylaxis immediately following the first injection of the second series.

Coincidently with these injections a fresh group of rabbits of similar average weight received the following intravenous inoculations:

Rabbit 11: Cholesterol colloid, 1 mg., and 0.25 mg. blood cell antigen in 2 c c distilled water.

Rabbit 12: Cholesterol colloid, 1 mg., and 1 mg. blood cell antigen in 2 c c water.

Rabbit 13: Cholesterol colloid, 1 mg., and 4 mg. blood cell antigen in 2 c c water.

Rabbit 14: Cholesterol colloid, 1 mg., and 8 mg. blood cell antigen in 2 c c water.

All animals were bled from the heart on the 5th day following the last injection and their inactivated serums tested as in Exper. 1.

Of the first series: Control Rabbit 3 serum showed 100% hemolysis.

Rabbits 1 and 7 serum showed 100% hemolysis.

Rabbits 2 and 8 serum showed 90% hemolysis.

Rabbits 4 and 5 serum showed 50% hemolysis.

Of the second series: Rabbits 11 and 14 serum showed 100% hemolysis.

Rabbit 13 serum showed 90% hemolysis.

Rabbit 12 serum showed 50% hemolysis.

These results were then checked and controlled by complement fixation and cross fixations as in the previous experiment, using, however, 3 antigens, one the blood cell fat antigen and two others differing slightly in strength only, having the composition noted in Exper. 3. From the results obtained it will be observed that the control rabbit had preserved its antibody strength, Rabbits 1 and 8 had gained, while all rabbits of the second series showed good antibody production.

In view of the fact that it had been possible in the case of the gonococcus to substitute an artificial product for the antigen derived from the germs,[4] it was determined to attempt an artificial substitute for the antigen of the red blood cells of the sheep. Accordingly, the total fatty acids from a large amount of thoroughly washed corpuscles were isolated and examined. The average values were as follows: Melting point, 78-80 C.; neutralization value, 140 KOH; iodin value, 70; calculated molecular weight, 400. This fat complex is somewhat unusual. The fatty acid corresponding most nearly in melting point and molecular weight was found to be cerotic acid with M. W. 396 and M. P. 78 C., but being a saturated acid has no iodin value. One apparently had to look then for a higher acid in combination with an unsaturated fatty acid of high iodin value. Such a combination, and probably the only one which would satisfy the requirements, was effected by combining melissic acid $C_{30}H_{60}O_2$, three parts, cerotic acid $C_{26}H_{52}O_2$, one part, and clupanodonic acid, $C_{18}H_{28}O_2$, an open chain acid having 8 unsaturated bonds, one part. This mixture approximates the melting point desired and has a theoretical M. W. of 405, and an iodin value of 73. Such a combination speaks for stability and high combining power. The melissic and cerotic acids were isolated from beeswax, and the clupanodonic acid was made from cod-liver oil by brominating the unsaturated fatty acids in ether, separating the octobromid and converting it into the acid by the action of zinc in HCl — alcohol, according to methods detailed by Lewkowitsch.[5] The sodium salts were then obtained from these acids and combined in the desired proportions.

Exper. 3.—Four rabbits of 2,000 gm. average weight received the following intravenous injections daily for 5 days:

Rabbit 15: Artificial blood cell antigen, 5 mg. in 2 c c water.

Rabbit 16: 3 c c typhoid suspension containing 5 mg. blood cell antigen.

Rabbit 17: Cholesterol colloid, 1 mg., and 1 mg. blood cell antigen in 2 c c water.

Rabbit 18: Cholesterol colloid, 1 mg., and 5 mg. blood cell antigen in 2 c c water.

The animals were bled 5 days after the last inoculation. Tests for the presence of antibody resulted as follows:

Rabbit 15, 100% hemolysis.
Rabbit 16, 90% hemolysis.
Rabbit 17, 75% hemolysis.
Rabbit 18, 90% hemolysis.

[4] Warden: Jour. Lab. and Clin. Med., 1916, I, p. 5; Jour. Am. Med. Assn., 1917, 68, p. 432.

[5] Chem. Technology of Oils, Fats and Waxes, 1914, I, p. 210 and p. 560 et seq.

Natural and artificial antigens gave identical readings both in hemolytic and complement fixation tests, as in the preceding experiments. There were no cross fixations with the antigens of gonococcus, treponema pallida, or pneumococcus. At this point it may be mentioned that all antigens have been controlled by fat salts derived from ordinary neutral fats such as lard, olive, cod-liver oil, coconut oil and palm oil.

From this experiment it is seen that the blood cell antigen has been approximated artificially, and that antibodies appear in the serum of rabbits injected with it.

Of the rabbits used in the preceding experiments there were selected the following for further observation, Nos. 1, 3, 8, 13, 14, and 15, these being in the best physical condition. It was observed that some had become cachectic during the process of immunization. After an interval of 14 days without injections these rabbits were again bled and the serums tested.

Rabbit 1 gave 50% hemolysis.
Rabbit 3 gave 50% hemolysis.
Rabbit 8 gave no hemolysis.
Rabbit 13 gave 100% hemolysis.
Rabbit 14 gave 75% hemolysis.
Rabbit 15 gave 75% hemolysis.

One week later, that is 3 weeks following the last inoculation, the rabbits referred to received intravenous inoculations daily for 4 days of 15 mg. artificial blood cell antigen and 2 mg. cholesterol colloid in 4 c c water. Rabbits 1 and 8 died immediately following the first of the injections. The remaining 4 animals were bled on the 5th day following the last injection and the serums tested for hemolytic power.

Rabbits Nos. 3, 13, 14, and 15, that is to say, all remaining animals, yielded serums which gave 100% hemolysis in doses of 0.04 c c of a 1:10 dilution. In Rabbits 13, 14, and 15 the serums possessed still higher titer, but its extent was not determined.

PRECIPITATION

Having shown that specific antibody can be produced in the serum of rabbits by injections of natural and pure artificial fat antigens in the form of sodium (or potassium) salts, it was determined to test whether such immune serums had the power to produce specific precipitation or flocculation when brought in contact with the antigen in the test tube. With the proportions by weight of antigen, cholesterol and serum employed in complement fixation as a guide the tests were undertaken first with water and salt solutions of the antigen, with such solutions in combination with aqueous cholesterol colloid, and

varying amounts of the serums to be tested. Without going into detail it was found that definite and specific precipitates were obtained with the aqueous solutions, but that it was difficult to obtain the opimum opacity of the mixtures with them and consequently difficult to get clear-cut readings. Alcoholic solution of the antigen and cholesterol was then substituted for the aqueous solutions. This alcoholic solution was the same as used for complement fixation, that is, 1 c c of the solution contained 0.002 gm. of artificial antigen and 0.008 gm. of cholesterol. With this antigen, salt solution and varying amounts of serum the tests were successful, giving complete clear-cut flocculations with settling of the precipitate and clearing of the fluid. A large number of tests were made from the data of which the following technic and protocol are given.

Into perfectly clean, small test tubes 1 by 8 cm., set in a convenient rack, there is pipetted 0.5 c c of salt solution. By means of a pipet so standardized as to drop a definite number of drops of the antigen to the gm., antigen is dropped squarely into the salt solution, the number of drops used being regulated by an optimum opacity of the resulting mixture after shaking. The mixture should be opalescent and "silky" by transmitted light and free from macroscopic particles. Too slight an opacity yields too small a precipitate while too dense an opacity may delay or prevent precipitation. From uniform pipets yielding drops of serum averaging 0.04 c c in quantity the control and determinant serums are dropped into the tubes containing the antigen and salt solution. All tubes are then thoroughly shaken. The method of dropping was found to be superior to that of allowing the substances to run down the inside of the tubes. The rack of tubes is then placed in the ice-box, but not in contact with ice, for 6-8 hours, or over night. Incubation at 37 C. in a water bath is not so satisfactory. At the expiration of this time the control tubes will show the same opacity as at the time of mixing while the positive tubes show complete flocculation, sedimentation and clearing. If the mixtures stand too long the control tubes of salt solution and antigen may show slight agglutination but without clearing or settling. It will be understood that all aqueous solutions of fat salts undergo slight changes with time owing to hydrolysis.

In the following tabulation the dose of antigen used, 6 drops, approximates $\frac{1}{16}$ gm. of the cholesterinized alcoholic antigen, or about 12 doses as employed in complement fixation, whereas the dose of serum varies from 2-4 times that used in complement fixation. In other words, the ratio of antigen to serum is about 3 times that used in the complement fixation test.

This antigen dosage represents about the maximum. With the same amount of salt solution, antigen doses of 4 drops gave equally good results but with a less amount of precipitate. The same results were obtained by using twice the quantity of salt solution and twice the

dosage of antigen and serum. When it is desirable to estimate the strength of the antibody titer 4 or more tubes may be used for each serum into which the doses of serum may be made to range from 0.02-0.32 or more.

TABLE 1

PRECIPITATIONS WITH ARTIFICIAL BLOOD CELL ANTIGEN

Each tube contains 0.5 c c salt solution and 6 drops of antigen + serum

0.12 c c salt solution	= —	0.24 c c typhoid serum	= —
0.24 c c salt solution	= —	0.12 c c normal rabbit serum	= —
0.12 c c normal human serum	= —	0.24 c c normal rabbit serum	= —
0.24 c c normal human serum	= —	0.12 c c Rabbit 3 serum	= +
0.12 c c meningococcus serum	= —	0.24 c c Rabbit 3 serum	= +
0.24 c c meningococcus serum	= —	0.12 c c Rabbit 13 serum	= ++
0.12 c c gonococcus serum	= —	0.24 c c Rabbit 13 serum	= ++
0.24 c c gonococcus serum	= —	0.12 c c Rabbit 14 serum	= ++
0.12 c c syphilitic serum	= —	0.24 c c Rabbit 14 serum	= ++
0.24 c c syphilitic serum	= —	0.12 c c Rabbit 15 serum	= ++
0.12 c c typhoid serum	= —	0.24 c c Rabbit 15 serum	= ++

The tubes were shaken after mixing and kept in the ice-box for 6 hours. The sign —, indicates no precipitation; +, partial precipitation, and ++, complete precipitation. It is not necessary to use control tubes containing salt solution and cholesterol alone because the latter is precipitated in the absence of the fat salt which tends to maintain it in high dispersion. In controlling these tests with normal human serum it was found occasionally that precipitation occurred. These serums were shown by other means to contain natural antisheep amboceptor.

THE GONOCOCCUS ANTIGEN

The gonococcus fat antigen, both natural and artificial, has been mentioned in earlier papers.[4] In order to determine whether antigens made from types of cells other than the animal red corpuscles would produce antibodies in the serums of animals injected with them the gonococcus artificial fat antigen was tested as follows:

Exper. 4.—Four rabbits, approximately 2,000 gm. in weight, received intravenous inoculations of antigen daily for 5 days.

Rabbit 19; gonococcus antigen, 8 mg. in 2 c c water.

Rabbit 20; typhoid suspension, 2 c c containing gonococcus antigen, 8 mg.

Rabbit 21; cholesterol colloid, 1 mg., and gonococcus antigen, 0.25 mg., in 2 c c water.

Rabbit 22; cholesterol colloid, 1 mg., and gonococcus antigen, 4 mg., in 2 c c water.

The rabbits, except No. 21 which died, were bled on the 5th day following the last inoculation and the serums tested.

Rabbit 19 gave 100% fixation.

Rabbit 20 gave no fixation.

Rabbit 22 gave no fixation.

For purposes of control the serums were also tested with syphilitic, pneumococcus, typhoid and red blood cell antigens with negative results. Control serums from normal rabbits, rabbits injected with cholesterol, rabbit antityphoid, normal human, syphilitic and gonococcus positive human serum were regularly negative with the gonococcus antigen except the last mentioned which was used as a positive control.

A fresh rabbit, No. 43, was substituted for Rabbit 21, and inoculations were resumed in Rabbits 20 and 22 and begun in 43. Commencing 9 days after the last previous injection each rabbit was inoculated daily for 3 days as follows:

Rabbit 19; no injection.

Rabbit 20; cholesterol colloid, 1 mg., and gonococcus antigen, 1 mg., in 2 c c water.

Rabbit 22; cholesterol colloid, 1 mg., and gonococcus antigen, 4 mg., in 2 c c water.

Rabbit 43; cholesterol colloid, 1 mg., and gonococcus antigen, 0.25 mg., in 2 c c water.

Twenty-four hours after the last injection the rabbits were bled. The serums reacted to complement fixation in the following manner:

Rabbit 19, no fixation.
Rabbit 20, 100% fixation.
Rabbit 22, 50% fixation.
Rabbit 43, 75% fixation.

Six days later, without further injections, the serum gave these results:

Rabbit 19, no fixation.
Rabbit 20, 50% fixation.
Rabbit 22, no fixation.
Rabbit 43, 50% fixation.

Two days later, 8 days after the last injection, Rabbits 20, 22 and 43 received on successive days 2 inoculations similar to those preceding. Two days thereafter their serums had changed again.

Rabbit 20, no fixation.
Rabbit 22, no fixation.
Rabbit 43, 100% fixation.

On the day following the last bleeding the same rabbits received the first of a final series of 5 daily injections of 20 mg. of antigen and 4 mg. of cholesterol in 3-4 c c of water. After an interval of 4 days the serums of Nos. 19, 22 and 43 were negative and the serum of No. 20 again gave 100% fixation. For convenience the tests are compared in the following table:

TABLE 2

COMPARATIVE SUMMARY OF A SERIES OF FIVE TESTS

Rabbit No.	Test 1	Test 2	Test 3	Test 4	Test 5
19	100%	Neg.	Neg.	Neg.	Neg.
20	Neg.	100%	50%	Neg.	100%
22	Neg.	50%	Neg.	Neg.	Neg.
43		75%	50%	100%	Neg.

These tests show that the injection of gonococcus antigen gives rise to a specific antibody in the serum of rabbits, but that the antibody is not great in amount and is transient in duration. This result is in accord with what takes place in the serum of man during the course of gonorrhea. It is well known by those experienced in the complement fixation test that the gonococcus antibody is often transient, coming and going during the progress of the disease. A negative test, therefore, may occur when gonococci are present and demon-

strable by smear and culture, and a single negative test, accordingly, may mean nothing. Repeated tests at intervals must be made before conclusions can be drawn. On the other hand, a single positive test is always of value. To illustrate: Of 58 prostitutes with gonorrhea under observation at the present time on whom single serum tests and many microscopic tests were made, 40 were positive to both smears and serum test, 12 were regarded as positive from smears alone in which the serum test was negative, and the diagnosis was established in 6 by the serum test in which smears failed.

Precipitation Test.—The precipitation test as described under the red blood cell antigen was applicable to the gonococcus fat antigen and antigonococcic serums when the latter were tested at the height of the antibody curve.

TABLE 3

SUMMARY OF RESULTS

To 0.5 c c salt solution and 4 drops of gonococcus antigen, serums were added with results as follows:

0.28 c c salt solution	= —	0.04 c c Rabbit 43 serum	= —
0.28 c c normal rabbit serum	= —	0.12 c c Rabbit 43 serum	= —
0.28 c c typhoid serum	= —	0.20 c c Rabbit 43 serum	= —
0.28 c c syphilis serum	= —	0.28 c c Rabbit 43 serum	= —
0.04 c c Rabbit 20 serum	= —	0.04 c c human serum	= +
0.12 c c Rabbit 20 serum	= +	0.12 c c human serum	= ++
0.20 c c Rabbit 20 serum	= +	0.20 c c human serum	= ++
0.28 c c Rabbit 20 serum	= ++		

PNEUMOCOCCUS FAT ANTIGEN

Having shown in the cases of the red blood cell of the sheep and of the gonococcus that specific antibodies against these cells are produced in the blood of rabbits by injecting the animals with the salts of the specific fats of the cells, similar experiments were made, with the fats of the pneumococcus to determine whether they possess antigenic power.

Antigens were prepared as follows: Mass cultures of Types 1, 2, and 3, separately and combined, were grown in fat free neutral beef-peptone-glucose broth in the presence of $CaCO_3$. At first, the germs were recovered by centrifugation but the process was found to be too slow when handling 20 or 30 liters of broth. Thereafter the 24 to 48-hour growths were saponified directly in the flasks by adding to each an excess of KOH and heating to 115 C. in the autoclave for one-half hour, after which the contents of the flasks were transferred to large evaporating dishes and concentrated to one-half or more volume on water baths under an air blast. The fluid was then made distinctly acid with sulphuric acid and brought to a boiling point after which it was transferred to tall beakers and solid sodium chlorid added nearly to saturation. The beakers were then set aside, after thorough stirring of the contents, under

glass covers until all the fatty acids had separated and risen to the surface, whereafter all the liquid except the top layer was carefully siphoned off and thrown away. From this small amount of residual fluid the acids were easily separated by ether and the remainder of the process was carried through in the usual way. The sodium salts of the fatty acids obtained from the individual types and from their combination were dissolved in alcohol in the proper concentration and tested for antigenic value against known antipneumococcus serums (New York State Health Laboratory and Rockefeller Institute).

The results of the tests may be briefly summarized: Fat antigen Type 1, gave perfect complement fixation with all three types of serum.

Fat antigen Type 2 gave perfect complement fixation with serums 2 and 3, and 50% fixation with Serum 1.

Fat antigen Type 3 gave the same fixations as Type 2.

The fat antigen from the mixed types was quite unsatisfactory, giving partial fixations only with Serums 1 and 3. There was, of course, no means of knowing whether all 3 types of cocci developed equally in the culture medium but it was inferred they did not do so. The difficulties encountered in isolating these antigens, due to the large bulk of culture medium necessary, led to a study of the fats in the hope that artificial antigens might be made. These artificial substitutes have been prepared and while they are still under consideration and undergoing tests it may be permitted here to summarize the results of complement fixation and immunization with them up to the present time.

An antigen to cover the 3 types of pneumococcus has been shown to give with the control serums of the 3 types complement fixation complete and equal with each, and no fixations with normal horse serum, or with syphilitic, gonococcus or red blood cell serums. With the serums of pneumonia patients in various stages of the disease there were few positive tests, and it was apparent that even about the time of the crisis the antibody quantity is seldom large and is transient. On the other hand, a certain number of persons appear to have antibody in their blood under normal conditions.

Exper. 5.—Four rabbits of 2,000 gm. average weight received the following intravenous injections of pneumococcus antigen daily for 5 days:

Rabbit 23, pneumococcus antigen 5 mg. in 3 c c water.

Rabbit 24, pneumococcus antigen 5 mg. in 3 c c typhoid suspension.

Rabbit 25, pneumococcus antigen 0.25 mg. and cholesterol colloid, 1 mg., in 3 c c water.

Rabbit 26, pneumococcus antigen 4 mg. and cholesterol colloid, 1 mg., in 3 c c water.

The rabbits were bled on the 5th day after the last inoculation. On testing the serums it was found that Rabbits 23 and 24 gave 100% fixation of complement, Rabbits 25 and 26 partial fixation. The normal and other controls were the same as with the gonococcus and red cell serums. This group of rabbits received a second series of injections of the antigen daily for 3 days, commencing on the 9th day after the last of the previous injections. The samples of blood were drawn after the expiration of 24 hours following the last inoculation of the series. The tests showed that the serums of Rabbits 24 and 26 gave 100% fixations of complement while Rabbits 23 and 25 gave no fixation whatever. Eight days later, without further injections all serums were negative except Rabbit 26 which still gave 100% fixation.

Two days later, that is 11 days after the last injection of the second series. Rabbits 23, 25 and 26 received a third series of injections, similar to the first,

on 2 successive days, and were bled after the expiration of 48 hours. The serums of all 3 animals gave complete fixations of complement. The following table summarizes the results:

TABLE 4
SUMMARY OF RESULTS OBTAINED IN EXPER. 5

Rabbit Number	Test 1	Test 2	Test 3	Test 4
23	100%	0	0	100%
24	100%	100%	0	Not tested
25	50%	0	0	100%
26	50%	100%	100%	100%

This experiment shows that specific antibody was produced in the blood of the animals injected with the fat antigen. It will be borne in mind that the antigen against which these tests were made was intended to be a general one and was not used as a means of identifying the strains. Only one agglutination test was made with Serums 24 and 26 controlled by normal rabbit serum, in which 0.5 c c of the serum, at the height of the antibody curve, was placed in contact with 0.5 c c of salt solution containing suspensions of Types 1, 2 and 3 from 24-hour blood-agar slants. Both serums agglutinated all 3 types in 2 hours at room temperature. As there was insufficient serum at the time for duplicate tests the procedure must be repeated.

The transient character of the pneumococcus antibody is as pronounced as in case of the gonococcus antibody. It is our observation also that the antibody in all antipneumococcic serums is not large in amount and is apt to be unstable. Those that have been kept cool and have been tested soon after inactivation react well but the antibody appears to deteriorate fairly rapidly after exposure to air, light and inactivation.

Precipitation Tests.—A series of tests were made on the precipitating properties of mixtures of pneumococcus antigen and antipneumococcus serums in salt solution in the same manner as was done with the red blood cell and gonococcus antigens and serums. The results of the tests with the general antigen and known serums are given in Table 5.

Slight alteration in the proportions of the ingredients does not interfere with the reaction as shown by using mixtures of 1 c c salt solution, 6 drops of antigen and graduated amounts of serum from 0.08-0.32 c c. The readings were practically identical. While the difference between the degrees of precipitation given by Serums 1 and 2 with the general antigen were so slight as to be detected with diffi-

TABLE 5

PRECIPITATION WITH ANTIPNEUMOCOCCUS SERUMS 1, 2, AND 3 AND PNEUMOCOCCUS ANTIGEN

In all cases 0.5 c c of salt solution and 5 drops of antigen were mixed with serum, etc.

0.04 c c salt solution	= —	0.16 c c pneumococcus serum 1	= ++
0.24 c c salt solution	= —	0.24 c c pneumococcus serum 1	= ++
0.04 c c normal horse serum	= —	0.04 c c pneumococcus serum 2	= ++
0.12 c c normal horse serum	= —	0.08 c c pneumococcus serum 2	= ++
0.24 c c normal horse serum	= —	0.16 c c pneumococcus serum 2	= ++
0.04 c c typhoid serum	= —	0.24 c c pneumococcus serum 2	= ++
0.12 c c typhoid serum	= —	0.04 c c pneumococcus serum 3	= ++
0.24 c c typhoid serum	= —	0.08 c c pneumococcus serum 3	= +
0.04 c c pneumococcus serum 1	= ++	0.16 c c pneumococcus serum 3	= +
0.08 c c pneumococcus serum 1	= ++	0.24 c c pneumococcus serum 3	= +

All tubes in ice-box, with occasional shaking, for 6 hours.
— indicates no precipitation; + partial, and ++ complete precipitation.

culty the precipitation with Serum 2 was the sharper and more clear-cut of the two. On the other hand, that given by Serum 3 was not so clearly defined as either of the others. This fact seemed to indicate that the antigen represented an average of slight variations in the proportions of the same fat constituents, and that by varying the proportions somewhat toward either extreme it would be possible to make antigens for Types 1, 2, and 3. This possibility had been indicated in earlier work on the fixation of complement with the antigen and the serums of the 3 types in which it was shown that variations of the same components of the fat antigen gave complete fixations with one type or another while the general type gave about equal fixations with all types. Accordingly the fatty ingredients of the antigen were combined in varying proportions above and below the average represented by the blanket type and were tested with the serums Types 1 and 3. The results showed that at certain points in these varying proportions precipitation became definite for the types and that beyond these points the reactions faded out. This showed fairly conclusively that the antigen types had been approximated. The results of the tests with Antigens 1 and 3 with the corresponding serums are shown in Table 6. Table 5 then illustrates the test of Type 2 antigen with its corresponding serum.

THE TYPHOID ANTIGEN

In a recent paper[1] it was shown that the fat complex peculiar to the typhoid bacillus gave specific complement fixation with antityphoid serum. By methods similar to those mentioned in connection with the red blood cell and other antigens a tentative artificial typhoid fat antigen of a general type has been worked out. The rather complex assortment of fats existing in the bacillus has made the antigen difficult

TABLE 6

PRECIPITATION, ANTIGEN TYPE 1 AND 3, SERUM TYPE 1 AND 3

Antigen Type 1	Antigen Type 3
0.04 c c serum Type 1............. = ++	0.04 c c serum Type 3............. = ++
0.08 c c serum Type 1............. = ++	0.08 c c serum Type 3............. = ++
0.16 c c serum Type 1............. = ++	0.16 c c serum Type 3............. = ++
0.24 c c serum Type 1............. = ++	0.24 c c serum Type 3............. = ++
0.04 c c serum Type 3............. = +	0.04 c c serum Type 1............. = +
0.08 c c serum Type 3............. = +	0.08 c c serum Type 1............. = +
0.16 c c serum Type 3............. = +	0.16 c c serum Type 1............. = +
0.24 c c serum Type 3............. = +	0.24 c c serum Type 1............. = +
0.32 c c serum Type 3............. = —	

The controls in each case are the same as in the preceding tests, and the tests were done in exactly the same manner.

to approximate, and up to the present time no effort has been made to produce separate antigens for the different antiserums of the members of the typhoid group. By means of the complement fixation test the antigen has been shown to possess group specificity for known serums of rabbits immunized with the bacilli of typhoid, paratyphoid A, paratyphoid B, and dysentery (Flexner), with the colon bacillus, and with serums of patients with typhoid fever which gave positive agglutination.

The power of this antigen to produce antibody in the blood of animals has been shown in the case of rabbits by injecting them intravenously with varying amounts of antigen in a manner similar to that used with other antigens, with the exception that suspensions of typhoid bacilli were not used, the surface action being supplied by cholesterol alone. The serums of the 4 rabbits so treated, controlled as in former tests, gave the following reactions at different periods during the course of the injections:

TABLE 7

SERUM REACTIONS OF FOUR RABBITS TREATED WITH TYPHOID ANTIGEN

Rabbit Number	Test 1	Test 2	Test 3	
4	50%	0	0	Fixation
31	100%	100%	50%	Fixation
32	0	100%	100%	Fixation
42	50%	0	0	Fixation

Rabbits 4, 31 and 32 had been used in previous experiments for purposes of control, having received injections of cholesterol colloid only. Rabbit 42 was a fresh animal.

Precipitation tests carried out as in previous experiments showed reaction to occur between the antigen and the known control antiserums of typhoid, paratyphoid A, paratyphoid B, colon and dysentery (Flexner) bacilli. Rabbit Serums 31 and 32 also gave positive reactions and produced agglutinations of suspensions of the members of the group with the exception of dysentery, which was not tried.

The facts brought out in the foregoing experiments in which it has been shown that certain fatty ingredients peculiar to four different

groups of cells give rise to specific antibody in the serums of animals appear to introduce a new phase into the problem of immunity. The work hitherto done on the relation of fats to antibody causation has dealt exclusively, so far as I am aware, with alcoholic extracts and so-called lipoids extracted by various solvents and the results have not been free from the criticism that the extractives contained protein. With the work under consideration, however, this criticism is only begging the question, and it seems to me that the pure fats must be recognized as playing a definite rôle in the immune processes. The response of the blood and cells of an animal that has been immunized with a protein in which a protective substance or state called antibody is induced has come to be recognized as definite, and to be fairly well understood in so far as the certainty of the results is concerned. This antibody is recognized by the delicate reactions of complement fixation and precipitation which occur when it is brought in contact in proper proportion with its antigen, and by certain well defined reactions, mild or fulminating, in the body of the animal. I believe we have shown that the same antibodies may be produced by fats alone and that they may be recognized by the same train of symptoms in the animal, by complement fixation, and by a delicate and specific precipitation test. It is just at this point at which the new phase referred to appears. The immunity conferred by protein alone and that produced by cells may be, and probably is, different. Protein is a constituent of cells. So is fat, but until now it seems as if the effort has been deliberately to disregard it or get it out of the way as calculated to confuse the question. Now the evidence from our experiments shows that the response to the fats is highly specific, whereas the same cannot be said for the proteins wherein specificity is confined more to type and less to species. The antibody against egg white from one source will react with egg white of another species, casein antibody responds to general casein antigens, and hemoglobin is hemoglybin from whatever source. The animal body, however, recognizes minute variations in the fat complexes such as are found in cells and is able to distinguish between related members of the same species, if not indeed individuals, as evidenced in the cases of the pneumococcus and the typhoid organisms.

In the problem the fat of the cell can be no more disregarded than its protein and should be recognized as one of the most important constituents. A study of the physiology and chemicophysics of cells leads

us to regard as fairly clearly established the function of the fats in maintaining at their surfaces a phase different from that in their interior and one instrumental in the regulation of surface energies and permeability. This matter has been discussed before,[6] and a most excellent review of the properties of cell surfaces is given by Bayliss.[7] It is well known that in certain bacteria and generally accepted that in nearly all cells the fats constitute a large but not necessarily exclusive part of their surfaces. When foreign cells invade the bodies of hosts it is precisely these surfaces which come in contact with the fluid colloids and exert influence on them, and much of the work of recent years points to the increasing importance of the effect of surface on plasma.

Proteins in aqueous solution possess surface and the same is true of fat salts in solution as shown not only by the opacity but also by their sensitiveness to the effects of electrolytes. The less soluble soaps in suspension in water produce death by anaphylaxis in rabbits quite as well as suspensions of agar or bacteria. Even the most soluble of the potassium and sodium salts of the fats develop surfaces on standing, owing to partial hydrolysis, or immediately on dilution with a large volume of water or salt solution yielding all transition stages from gel to sol and to suspensoid. It may be objected that there is no evidence to show that the salts of the fat antigens are in the same state as the fats exist in the cells. This objection can hardly be sustained in view not only of the evidence submitted here, but also of the fact that cells are rich in soaps and potassium which emulsify all other forms of fats and which are of all forms the most powerful agents in lowering surface tension. With the continued presence in the body of the host of living invading cells or with their destruction within the tissues a particular fat complex is probably continually in contact with the plasma in much the same form as in the antigens.

Each bacterial cell appears to have fat peculiar to itself which as a whole constitutes a definite complex or entity, or configuration. Chemists would call it a eutectic mixture. Group relationship in bacteria such as the types of pneumococci and the typhoid bacilli is characterized as to fats by minute variations of the same constituents, and these variations in themselves constitute definite complexes. Organisms wide apart in species as for instance the gonococcus, the

[6] Warden: Jour. Am. Med. Assn., 1917, 68, p. 432.
[7] Principles of Physiology, 1915.

tubercle bacillus, and the typhoid bacillus have fats wholly dissimilar. In the former case the differences are not so great but that the tests of complement fixation and precipitation differentiate the antigens narrowly, whereas in the latter case there are few or no cross fixations. But it has been observed that when antigens though wide apart as to configuration nevertheless contains an excess of one or more ingredients in common there is bound to be some overlapping as shown by occasional partial fixation or precipitin reactions. This has seemed to us to offer a partial explanation for certain partially positive reactions occurring with the syphilitic and other antigens otherwise unaccountable, but other conditions of which we know nothing may contribute to this phenomenon.

The plan of the antigen injections discussed in connection with the experiments in which it was sought to compare the effects of immunizing with pure antigen and with adjuvants having greater surface has led to no definite conclusions. Antibodies appeared as readily, apparently, to the one as the other, but in all the forms of injection it was impossible to avoid the factor of surface. It seems, however, that the best results were had from the combinations of antigen and cholesterol.

The question of the duration or permanence of the antigen-induced antibody has not been gone into fully, but it is apparent in the instances of the red blood cell and the typhoid bacillus that it does not persist as long in the body of the animal as that induced by immunization with the entire cell. There have been, however, during the period covered by experiments undoubted instances of sensitization as shown by anaphylactic signs more or less severe appearing in animals on reinjecting them after a considerable time was allowed to pass. This was noticed also on injecting animals and man with protein-free syphilitic antigen in experiments not yet reported.

The precipitation of mixtures of fat antigens and antiserums is regarded as a delicate and specific reaction, fully as much so as the precipitin test with protein antigen. While complement is unnecessary in such mixtures there is no question but that perfectly fresh serums give clearer reactions than those that have been inactivated or allowed to stand.

The question of total specificity in immunity it seems must include no one group of substances in the infecting cell. Rather must the antigen be inclusive of all determining substances. With the knowl-

edge that antibody to protein is persistent and represents broadly the type of the antigen, that the antibody produced by cells possesses more or less persistence together with high specificity for those cells, and that in general the antibody induced by fat antigens resembles that from the cells in all particulars, except possibly in degree of persistence, it may be inferred that the specificity of such antibody is in part or wholly due to the fats of the cell. Some bacterial cells such as the typhoid, spirochetes and others lead to long immunity with specificity confined to a group, while other cells, like the pneumococcus, gonococcus, meningococcus and streptococcus, yield at best a transient immunity but sharply defined specificity which makes it appear as though the fault apparently inherent in the family of coccaceae were attributable to the protein, especially since it has been possible to produce with fat antigen a higher antibody titer than has been met in actual disease and equally as high as that attained by injections of the cells.

CONCLUSIONS

The fat complexes characteristic of certain bacteria and other cells obtained either from the cells or assembled artificially are capable of replacing the cells themselves in the production of specific antibodies in the blood of rabbits injected with them.

The specificity of the antibodies obtained by the injection of cells probably depends in part or wholly on the configuration of the fats constituting the bulk of the cell surfaces.